MATERNAL FETAL MEDICINE/BIOLOGY: ORIGINAL ARTICLE

Longitudinal Assessment of Oxidative Stress Markers in Women with Preeclampsia

Aditi Godhamgaonkar[1] · Kamini Dangat[1] · Karuna Randhir[1] · Sunaina Chhetri[1] · Kajal Shelke[1] · Divya Shukla[1] ·
Girija Wagh[2] · Sanjay Lalwani[2] · Sanjay Gupte[3] · Sadhana Joshi[1]

Abstract

Preeclampsia (PE) is a pregnancy-specific disorder and a major contributor to maternal and fetal morbidity and mortality. Role of oxidative stress in early pregnancy with the pathophysiology of the disorder is unclear. The current study aims to analyse maternal levels of oxidative stress markers (MDA and protein carbonyl) longitudinally across gestation and placental levels of oxidative stress markers (MDA, protein carbonyl and 8-oxo-2'-deoxyguanosine) in women with PE and compare them with non-PE women. 324 pregnant women (216 non-PE and 108 PE women) were longitudinally followed during pregnancy. Women with preeclampsia were stratified as early onset preeclampsia (EOP) and late onset preeclampsia (LOP) Maternal blood at four time points across gestation (11–14 weeks, 18–22 weeks, 26–28 weeks, and at delivery) and placenta were collected. Maternal and placental levels of oxidative stress markers were assessed using commercially available kits. Maternal plasma MDA and protein carbonyl levels were comparable between the PE and non-PE group at all timepoints across gestation. Maternal plasma MDA were significantly higher levels at 26–28 weeks in EOP women when compared to non-PE women ($p < 0.05$). Placental 8-oxo-dG levels were lower in the EOP group as compared to non-PE ($p < 0.05$). Elevated plasma MDA levels were positively associated with birth length at 18–22 weeks and 26–28 weeks in the PE group ($p < 0.05$ for both). Maternal plasma MDA levels were positively associated with systolic blood pressure at 18–22 weeks. Oxidative stress in early pregnancy is not associated with risk of PE.

Keywords Oxidative stress · Malondialdehyde · Protein carboxyl · 8-Oxo-2'-deoxyguanosine · Preeclampsia · Pregnancy

Introduction

Preeclampsia (PE) is a multifactorial disorder and chief contributor to maternal and fetal morbidity and mortality with a worldwide prevalence of 3–12% [1]. PE can be classified according to the onset-time of clinical symptoms as early-onset PE (EOP) or late-onset PE (LOP) [2] both of which are different entities associated with different clinical features

and pathophysiology [3]. EOP is believed to be associated with abnormal placentation and impaired spiral artery remodeling [4]. The impaired spiral artery remodeling leads to uteroplacental hypoxia/reoxygenation triggering oxidative stress and placental apoptosis [5]. On the other hand, LOP results due to a mismatch between the metabolic demands of the fetus and the maternal supply [6]. The progressive mismatch between maternal perfusion and fetoplacental demands induces oxidative changes in LOP placentae [3].

Physiological levels of reactive oxygen species (ROS) are required for normal progression of placental and fetal growth and development. A balance between oxidants and antioxidants is crucial for fertilization, implantation and placental growth [7]. Increased oxidative stress, induced by ROS arises from an imbalance between the generation of oxidants and their clearance by antioxidants [8–10]. Excess production of oxidants may lead to distorted signaling and damage to cellular/molecular structures like lipids, proteins and DNA [8, 11].Common oxidative stress markers include

✉ Sadhana Joshi
sadhana.joshi@bharatividyapeeth.edu

1 Mother and Child Health, Interactive Research School for Health Affairs, ICMR- Collaborating Centre of Excellence (ICMR-CCoE), Bharati Vidyapeeth (Deemed to Be University), Pune Satara Road, Pune 411043, India

2 Department of Obstetrics and Gynaecology, Bharati Medical College and Hospital, Bharati Vidyapeeth (Deemed to Be University), Pune 411043, India

3 Gupte Hospital and Research Centre, Pune 411004, India

lipid peroxidation product malondialdehyde (MDA), protein oxidation product such as protein carbonyls and DNA damage products such as 8-Oxo-2'-deoxyguanosine (8-oxo-dG) [12]. Earlier studies by us and a few others have demonstrated increased levels of maternal MDA in women with PE at the end of pregnancy [13–19]. Similarly, several authors demonstrate elevated levels of protein carbonyls in the third trimester of pregnancy and at the time of labor in PE pregnancies as compared to healthy pregnancies [20–25]. However, these findings were observed after the progression of the disorder making it difficult to understand the involvement of oxidative stress in the pathophysiology of the disorder. There are limited studies that have investigated the levels of oxidative stress markers in pregnancies complicated with preeclampsia throughout the gestation but these studies report conflicting results [26–28]. However, these studies are limited by small sample size and did not examine the changes in oxidative stress indices in subtypes of PE.

In view of this, the current study analyzed maternal levels of oxidative stress markers (MDA and protein carbonyl) at four different timepoints across gestation in women preeclampsia and their subtypes and compares them with non-PE women. Moreover, the study also analyzed placental levels of oxidative stress markers (MDA, protein carbonyl and 8-oxo-dG) in the above women.

Materials and Methods

Study Subjects

The current work is a part of a large ongoing study funded by the Indian Council of Medical Research as a Centre of Advanced Research (ICMR-CAR) – "Investigating Mechanisms leading to Preeclampsia" at IRSHA, Bharati Vidyapeeth University, Pune (5/7/1069/13-RCH, dated 31–03-2017). Bharati Vidyapeeth Medical College Institutional Ethical Committee approved the study protocol (IEC/2015/37, dated 03.10.2015). Pregnant women visiting Bharati Medical College and Hospital, and Gupte Hospital and Research Centre, Pune, for their first antenatal visit were approached by the counselor and their participation was solicited, those who agreed to participate and were ready to deliver at the above hospitals were recruited in the study and followed up throughout their gestation till delivery. Written informed consent was obtained from the women. Each woman's personal, obstetric, clinical and family history were recorded. The detailed study design and recruitment criteria has been discussed earlier [29].

Briefly, pregnant women were recruited at first antenatal visit and followed up at four different timepoints of gestation i.e., 11–14 weeks of gestation (V1), 18–22 weeks (V2), 26–28 weeks (V3) and at delivery (V4). All women were

followed until delivery and categorized as PE if they developed hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg on at least two measurements taken at least 4 h apart) after 20 weeks of gestation along with new onset of one of the followings: proteinuria thrombocytopenia, impaired liver function, development of renal insufficiency, pulmonary edema, or new-onset cerebral or visual disturbances. PE women were categorized as EOP if they developed PE before 34 weeks of gestation whereas if the women developed PE later than 34 weeks of gestation they were categorized as LOP.

A total of 1154 women were recruited in the study at 11–14 weeks of gestation, among whom 1096 had singleton pregnancies and 58 had twin pregnancies. Of the 1096 singleton pregnancies, 108 developed preeclampsia. We selected control and cases in a ratio of 2:1. For each case of preeclampsia, 2 non-PE women delivering at the same gestational age (± 4 gestational weeks) were randomly selected using "Select Cases" in SPSS. Therefore, the current study investigated changes in oxidative stress markers in 324 women included in the study (108 who developed Preeclampsia and 216 non-Preeclampsia women).

Sample Collection and Processing

Maternal blood was collected into vacutainers containing EDTA at four different timepoints (V1, V2, V3 and at delivery) for each participant. The blood was processed to separate serum, plasma, erythrocyte and lymphocyte fractions of blood that were stored at -80 °C until further analysis.

Fresh placental tissue was obtained immediately after delivery. The fetal membrane was trimmed off and the umbilical cord cut 2 cm away from the insertion point. One-third radius area around cord insertion site was considered the region of interest and cut. From this region, basal and chorionic plate were cut and discarded. Small tissue pieces were made from this region. The small pieces were then placed in cryovials, snap frozen in liquid nitrogen and then stored at -80 °C [29].

Preparation of Placental Tissue Homogenate and Estimation of Total Protein

For MDA analysis, 1 ml of 1X phosphate buffered saline (PBS) was used to homogenize 300 mg of placental tissue. Protease inhibitors (leupeptin, aprotinin and phenyl methane sulphonyl fluoride) were added to the buffer. To lyse the cell membrane in the homogenized tissue two freeze–thaw cycles were given. The homogenate was then centrifuged at 5590×g for 10 min at 4ºC and the supernatant was collected and stored at -80ºC.

For protein carbonyl estimation, 1 ml of cold buffer was used to homogenize 300 mg of placental tissue {cold

buffer: 50 mM PBS [sodium chloride (NaCl) 8.76 g; potassium dihydrogen phosphate (KH2PO4) 3.22 g; dipotassium hydrogen phosphate (K2HPO4) 4.58 g; deionized water up to 1L; pH 6.7] with 1 mM EDTA}. The homogenate was then centrifuged at $5590 \times g$ for 15 min at 4ºC and the supernatant was collected and stored at -80ºC.

The supernatant was then assayed for total protein content using Pierce™ BCA Protein Assay Kit (Cat. No: 23225). The oxidative stress marker levels were adjusted to total protein concentration in the samples.

Biochemical Estimation

Maternal MDA level

MDA levels were measured from maternal plasma and placenta using Aoxre LLC Oxis Research™ Bioxytech® MDA-586™ Spectrophotometric Assay (Cat. No: 21044). Maternal plasma MDA was expressed as μM and placental MDA was expressed as μM/mg.

Maternal Protein Carbonyl levels

Plasma protein carbonyl levels were measured using Cell-BioLabs' Oxiselect™ Protein Carbonyl Elisa kit (Cat. No: STA-310) and expressed as nmol/mg. Placental protein carbonyl was measured using Cayman Chemical's (USA) Protein Carbonyl Colorimetric assay kit (Cat. No: 10005020) and expressed as nmol/mg.

Placental 8-oxo-dG levels

Placental 8-oxo-dG levels were measured using 8-hydroxy 2 deoxyguanosine ELISA kit by Abcam, USA (Cat. No: ab201734) and expressed as ng/μg of DNA. DNA from placental tissue was extracted using Qiagen Blood & Tissue Kit following standardized protocol for human tissue. dsDNA was denatured by heat shock to yield ssDNA. ssDNA was digested by nuclease P1 and was then treated with alkaline phosphatase. The treated DNA samples were then assayed for 8-oxo-dG following the manufacturer's protocol.

Statistical Analysis

SPSS/PC + package software (Chicago, IL, USA) was used to analyze the data (Version 28.0). Continuous variables were expressed as Median [Interquartile range (IQR)] and adjusted for gestational age. Log transformation was used for skewed variables. Univariate associations were tested between oxidative stress markers and potential confounders: maternal age, gestational age, body mass index (BMI), standard of living index (SLI) score, hospital, gestational diabetes mellitus (GDM). Multiple linear regression was used to examine the association of maternal MDA with different parameters after adjusting for maternal age.

Results

Maternal and Neonatal Characteristics

Women with PE had a higher maternal BMI (28.0 ± 5.4 kg/m^2) as compared to non-PE women (24.1 ± 4.1 kg/m^2) at recruitment ($p < 0.01$). Systolic [non-PE: (108.6 ± 8.4 mmHg); PE: (116.5 ± 11.4 mmHg)] and diastolic blood pressure [non-PE: (70.3 ± 6.6 mmHg); PE: (75.2 ± 8.4 mmHg)] at recruitment was higher in women with PE as compared to non-PE women ($p < 0.01$) [30]. Systolic and diastolic blood pressure remained higher in women with PE as compared to non-PE women throughout gestation and has been reported by us earlier [30]. The mean gestational age at which PE was clinically diagnosed was 34.3 weeks. Birth weight was lower in PE (2864.5 ± 422.4 gm) as compared to the non-PE group (2698.4 ± 627.2 gm) ($p < 0.01$) [30].

Maternal plasma MDA levels

MDA levels in women with PE were comparable to non-PE women (Fig. 1).

When these levels were assessed in subtypes of PE i.e., EOP and LOP showed that MDA levels in women with EOP were higher at V3 timepoint ($p < 0.05$) than non-PE women (Fig. 1). Maternal plasma MDA levels were not associated with risk of PE in in adjusted and unadjusted model (Table 1).

Maternal Plasma Protein Carbonyl levels

Maternal plasma protein carbonyl levels were higher in preeclampsia group but were not significant (Fig. 2).

Plasma protein carbonyl levels were comparable between the subtypes of PE (Fig. 2). Maternal plasma protein carbonyl levels were not associated with risk of PE in in adjusted and unadjusted model (Table 1).

Placental MDA, Protein Carbonyl and 8-oxo-dG levels

Placental oxidative stress markers were comparable between the PE and non-PE groups (Fig. 3). When the levels were assessed in the subtypes of PE, placental 8-oxo-dG levels were significantly lower in the EOP group as compared to non-PE (Fig. 3).
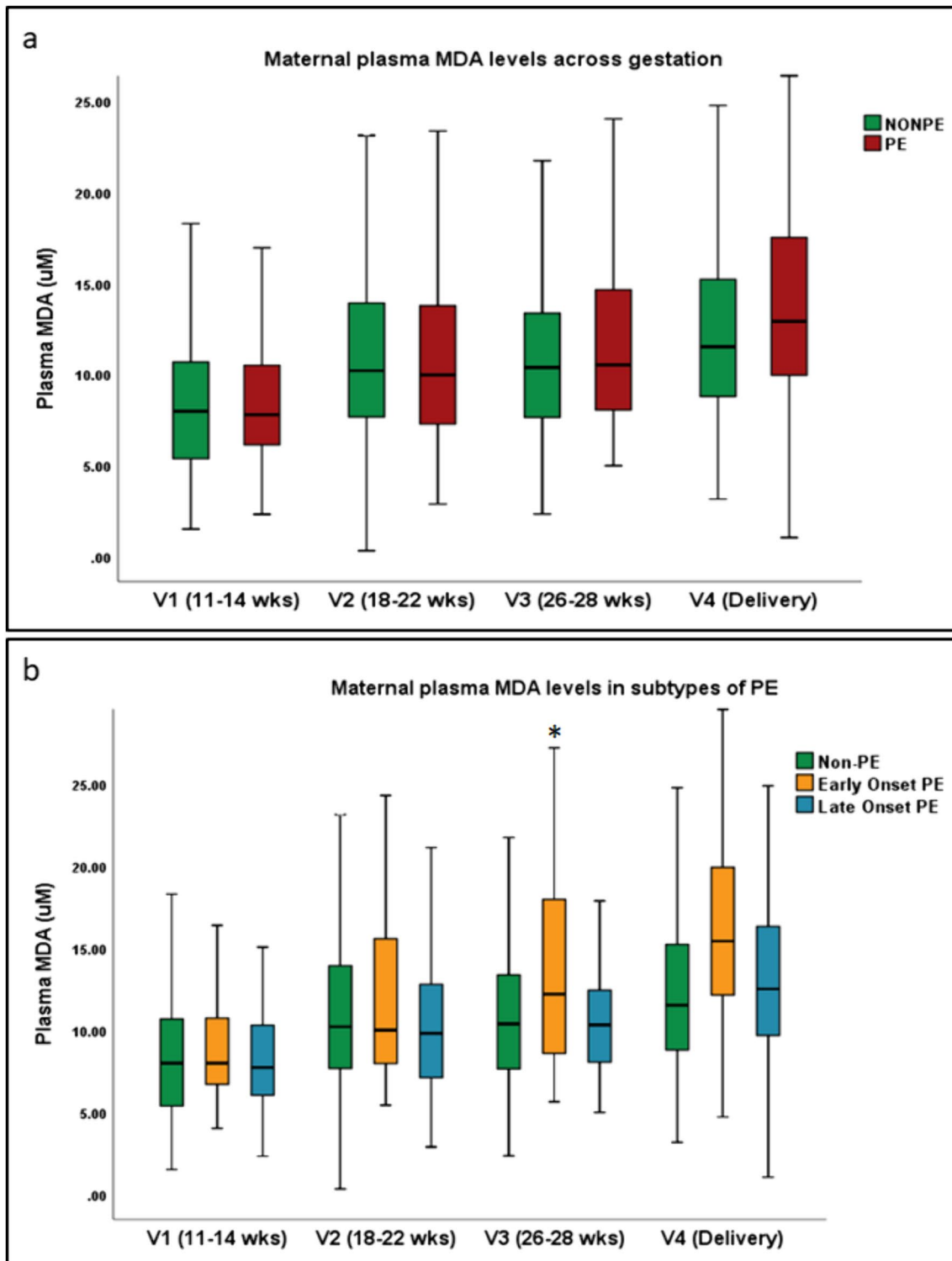
Figure 1 Maternal plasma MDA levels. Boxplot representing maternal plasma MDA levels across gestation. **a** Maternal plasma MDA levels across gestation **b** Maternal plasma MDA levels in subtypes of PE. PE: Preeclampsia; EOP: Early Onset Preeclampsia; LOP: Late Onset Preeclampsia

Table 1 Association of maternal oxidative stress markers levels with risk of PE

Oxidative Stress markers	Time Points	n	OR Unadjusted model	p	OR Adjusted model	p
MDA (µM)	V1	306	1.001 (0.95, 1.07)	0.977	0.997 (0.93, 1.08)	0.894
	V2	289	0.999 (0.96, 1.05)	0.937	0.990 (0.94, 1.05)	0.697
	V3	289	1.03 (0.99, 1.09)	0.225	1.04 (0.99, 1.11)	0.148
	V4	284	1.03 (0.99, 1.08)	0.150	1.03 (0.99, 1.08)	0.210
Protein carbonyl (nmol/mg)	V1	98	1.01 (0.73,1.39)	0.988	0.88 (0.59,1.3)	0.504
	V2	94	1.08 (0.8,1.47)	0.643	1.15 (0.81,1.61)	0.451
	V3	95	1.25 (0.77,2.01)	0.378	1.71 (0.94,3.12)	0.080
	V4	96	0.98 (0.74,1.29)	0.838	1.05 (0.76,1.46)	0.791
	PLC	193	1.22 (0.67,2.21)	0.520	1.35 (0.71,2.57)	0.364
8-oxo-dG (ng/µg DNA)	PLC	249	0.44 (0.07,2.93)	0.393	0.34 (0.04,2.75)	0.306

[a]: adjusted for age, BMI, and SLI; OR: Odds Ratio; CI: 95% Confidence Interval; n = number of samples; p = level of significance

Association of Maternal Oxidative Stress Markers with Blood Pressure and Birth Outcome

Maternal plasma MDA levels were positively associated with systolic blood pressure at V2 ($p < 0.01$) timepoint. However, there was no association between systolic blood pressure and plasma MDA levels at V1, V3 and V4 timepoints (Table 2).

There was no association between diastolic blood pressure and plasma MDA levels at any timepoint (Table 2).

There was no association between systolic and diastolic blood pressure and plasma protein carbonyl levels at all timepoints (Table 2).

Maternal plasma MDA levels at V1 and V3 and placental levels were positively associated with birth length ($p < 0.05$) (Table 3).

There was no association of protein carbonyl levels and placental 8-oxo-dG levels with birth weight and birth length (Table 3).

Discussion

The present study for the first time gives an enlarged view of the circulatory oxidative stress measures of pregnant women diagnosed with PE, as it sought to examine the changes across the gestation beginning as early as 11–14 weeks of gestation.

The key findings of the study are: 1) Maternal plasma MDA and protein carbonyl levels were comparable between the PE and non-PE group at all timepoints across gestation. 3) When the levels of OS markers were analyzed in subtypes of PE, maternal plasma MDA were significantly higher at 26–28 weeks in women with early onset PE but not late onset when compared to non-PE women. 4) Maternal plasma MDA levels at 18–22 weeks was associated with systolic blood pressure. 5) None of the markers were associated with the risk of preeclampsia 6) Maternal plasma MDA levels was positively associated with birth length.

The current study demonstrates that maternal plasma MDA levels in early pregnancy were not higher in women with PE as compared to normotensive control women. MDA is a byproduct of lipid peroxidation which is known to induce endothelial dysfunction, damage cell membranes and reduce endothelial vasorelaxation [1]. Defective placentation due to inadequate remodeling of uterine spiral arteries induces endothelial dysfunction in PE. Although emerging evidence implies the involvement of increased oxidative stress in PE, it is still unclear whether this endothelial dysfunction caused due to oxidative stress is implicated in pathogenesis of the disorder or is it merely a malfunction instigated by establishment of the disorder which may lead to increased oxidative stress.

Increased lipid peroxidation has been linked with pathogenesis of PE. To support this hypothesis, many studies have reported increased plasma MDA levels in women with PE compared to normal healthy women at 24–36 weeks [28, 31] and in the third trimester [18]. Additionally, only one study has examined the changes in levels of maternal MDA levels based on severity which suggests that higher blood levels of MDA at delivery correlate with severity of PE [32]. Further few studies report higher maternal blood levels after 20 weeks of gestation in women with PE [18, 33].
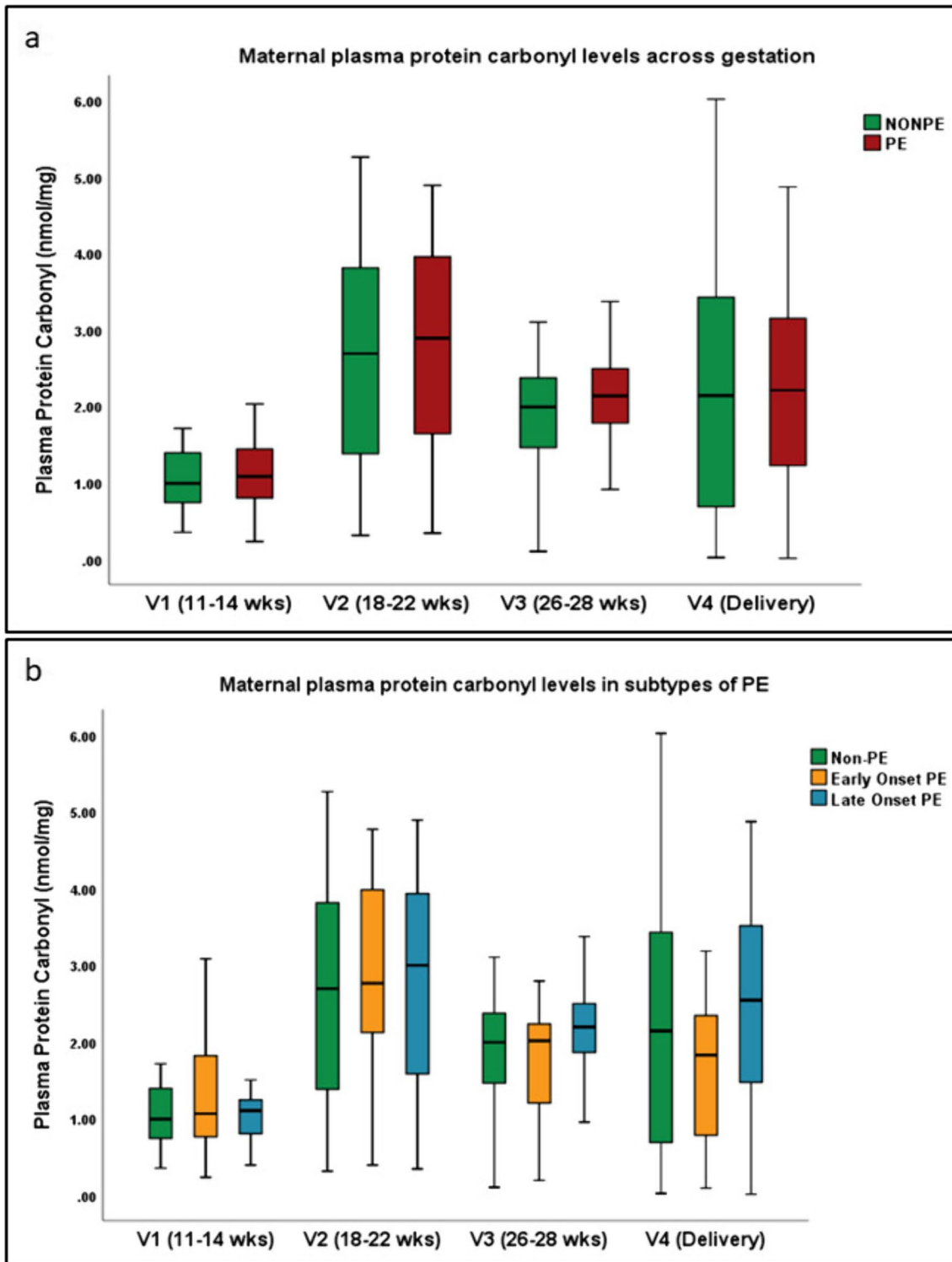
Figure 2 Maternal plasma protein carbonyl levels. Boxplot representing maternal plasma protein carbonyl levels across gestation. **a** Maternal plasma protein carbonyl levels across gestation **b** Maternal plasma protein carbonyl levels in subtypes of PE. PE: Preeclampsia; EOP: Early Onset Preeclampsia; LOP: Late Onset Preeclampsia; p: the level of significance; *p < 0.05 as compared to non-PE
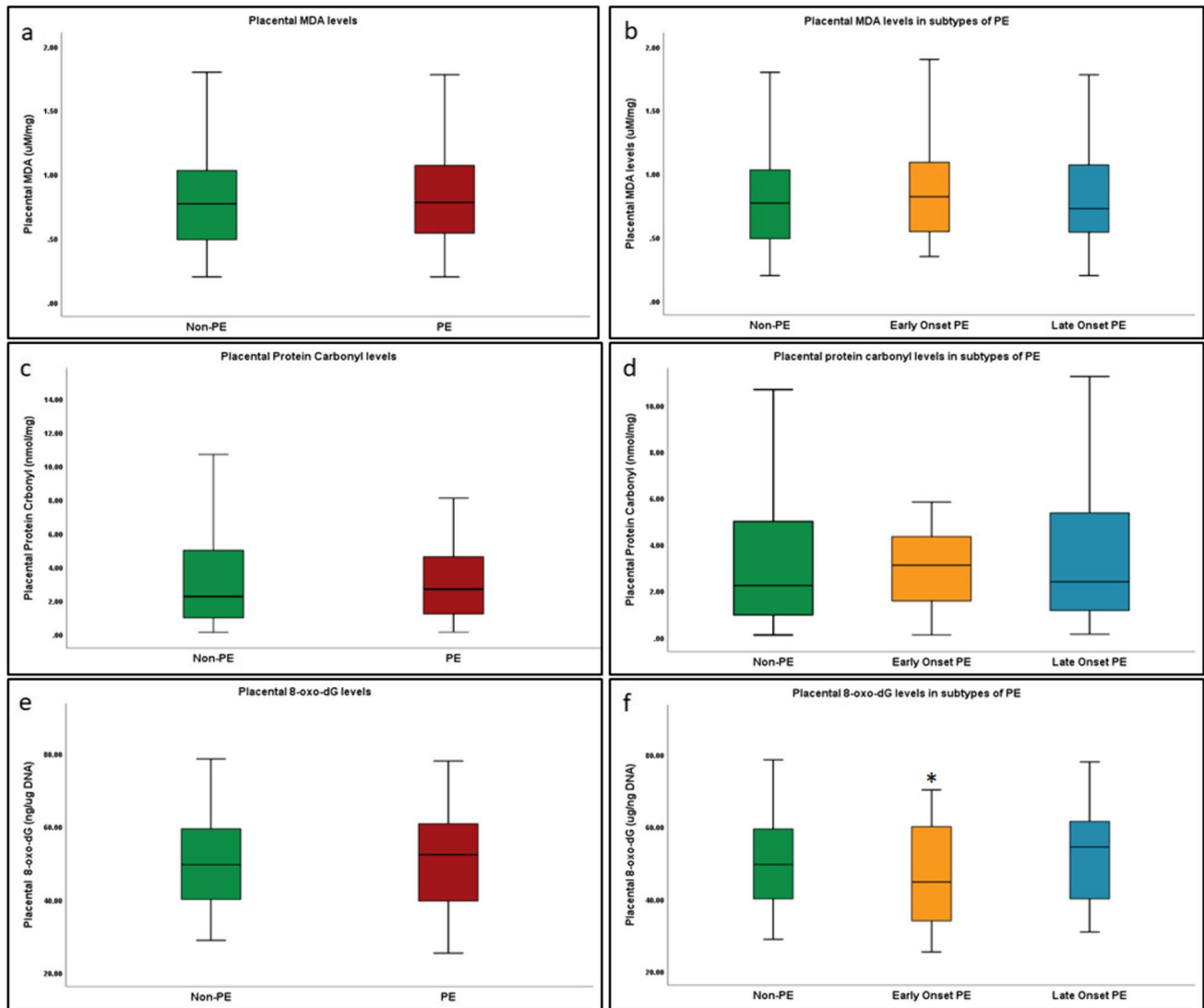
Figure 3 Placental levels of oxidative stress markers. Boxplot representing placental levels of oxidative stress markers. **a** Placental MDA levels (PE vs. Non-PE) **b** Placental MDA levels in subtypes of PE **c** Placental protein carbonyl levels (PE vs. Non-PE) **d** Placental protein carbonyl levels in subtypes of PE **e** Placental 8-oxo-dG levels (PE vs. Non-PE) **f** Placental 8-oxo-dG levels in subtypes of PE. PE: Preeclampsia; EOP: Early Onset Preeclampsia; LOP: Late Onset Preeclampsia; p: the level of significance; *p < 0.05 as compared to non-PE

The present study reports increased levels of plasma MDA after the onset of PE at 26–28 weeks of gestation but not in early pregnancy. This suggests that the increase in oxidative stress is successive to the disorder. In contrast, a study conducted to examine the levels of MDA from early pregnancy on a small sample size of 21 preeclamptic women and 24 control women has reported higher serum MDA levels between 10–12 weeks and 20–24 weeks of gestation in women with PE compared to normotensive control women [27]. Additionally, our earlier longitudinal study has reported increased plasma MDA levels at 16–20 weeks of gestation in PE pregnancies as compared to control [26]. This discrepancy could be attributed to the difference in the

time points as well as socio-economic status of the women studied.

The results of this study show that oxidative damage, which was evaluated by protein carbonyls in plasma, was unchanged in PE. This is in accordance with other studies that have shown that carbonylation in third trimester can be unaltered in PE compared with normal pregnancy [34, 35].

The present study also reports no change in the levels of placental 8-oxo-dG in PE women as compared to women in the non-PE, although it was lower in the EOP group. However, this is contradictory to studies that have reported increased oxidative DNA damage measured by placental 8-oxo-dG levels in women with PE [36–39] where severe

Table 2 Association of maternal MDA levels with blood pressure

	Time Point	Unadjusted Model			Adjusted Model		
		n	B (CI)	p	n	B (CI)	p
MDA (µM)	MDA association with systolic BP						
	V1	305	-0.01(-0.3, 0.28)	0.959	305	-0.19 (-0.47, 0.1)	0.193
	V2	288	0.38 (0.19, 0.58)	0.001	288	0.32 (0.12, 0.52)	0.002
	V3	287	0.09 (-0.18, 0.35)	0.514	287	-0.01 (-0.26, 0.25)	0.970
	V4	284	0.19 (-0.05, 0.42)	0.120	283	0.09 (-0.15, 0.33)	0.463
	MDA association with diastolic BP						
	V1	305	-0.06 (-0.27, 0.16)	0.620	305	-0.18 (-0.39, 0.05)	0.111
	V2	288	0.10 (-0.05, 0.25)	0.193	288	0.03 (-0.12, 0.18)	0.701
	V3	287	0.05 (-0.14, 0.23)	0.614	287	-0.03 (-0.20, 0.16)	0.820
	V4	284	0.07 (-0.11, 0.24)	0.454	283	0.05 (-0.14, 0.23)	0.605
Protein carbonyl (nmol/mg)	Protein carbonyl association with systolic BP						
	V1	98	-4.03 (-11.55, 3.5)	0.291	98	-2.87 (-9.69, 3.96)	0.406
	V2	94	2.08 (-5.28, 9.42)	0.577	94	-0.30 (-6.48, 5.89)	0.924
	V3	95	-0.48 (-8.00, 7.06)	0.901	95	-1.93 (-9.44, 5.59)	0.612
	V4	96	1.65 (-3.76, 7.06)	0.546	96	1.51 (-3.55, 6.55)	0.556
	Protein carbonyl association with diastolic BP						
	V1	98	0.40 (-4.40, 5.20)	0.869	98	0.89 (-3.29, 5.06)	0.673
	V2	94	0.23 (-4.93, 5.39)	0.930	94	-0.34 (-5.37, 4.70)	0.896
	V3	95	-0.16 (-5.19, 4.88)	0.952	95	-1.44 (-6.53, 3.66)	0.577
	V4	96	0.09 (-3.92, 4.09)	0.966	96	-0.34 (-4.19, 3.51)	0.862

Adjusted for age, BMI, SLISCORE, incidence of PE and GDM and the participating hospitals; BMI: Body Mass Index; BP: Blood Pressure; SLISCORE: Standard of Living Index Score; n = number of samples; p = level of significance

Table 3 Association of maternal oxidative stress markers with birth outcome

Oxidative Stress Markers	Time Points	Birth Weight			Birth Length		
		n	r	P	N	R	p
MDA (µM/mg)	V1	305	0.064	0.266	282	0.119	0.047
	V2	288	-0.080	0.177	267	0.074	0.233
	V3	288	0.045	0.449	270	0.144	0.019
	V4	284	-0.052	0.385	264	0.089	0.150
	Placental	312	0.046	0.421	290	0.135	0.022
Protein carbonyl (nmol/mg)	V1	97	-0.040	0.702	86	0.057	0.610
	V2	93	-0.019	0.863	83	-0.141	0.216
	V3	94	-0.014	0.896	84	-0.071	0.532
	V4	95	-0.051	0.633	85	-0.057	0.616
	Placental	192	0.063	0.393	179	-0.072	0.344
8-oxo-dG (ng/µg DNA)	Placental	248	-0.052	0.419	229	-0.080	0.230

All values are adjusted for BMI, age, baby gender, SLISCORE and group; n = number of samples; r = Pearson's correlation; p = level of significance

damage was observed in women with EOP [40]. All the above-mentioned studies were conducted on a smaller sample size and have used immunohistochemistry to measure the proportion of oxidative DNA damage. Further studies are needed to clarify the role of placental 8-oxo-dG in the pathophysiology of PE.

The current study reports positive association of maternal plasma MDA levels at 11–14 weeks and 26–28 weeks with baby length. A study conducted on Bangladeshi healthy pregnant women aiming to evaluate the associations of oxidative stress markers in late pregnancy with infant size reported that higher levels of lipid peroxidation markers at

14 weeks of gestation were associated with larger infant size (baby length and chest circumference) [41]. Other studies have reported no association of maternal plasma MDA with birth length [26, 42]. A recent study reports no association of newborn birth length with placental MDA levels [43].

The current study demonstrates maternal MDA levels in early pregnancy to be associated with increased systolic BP. A study examining the oxidative stress levels in serum of 50 PE pregnancies also reported a positive association of maternal serum MDA levels in late third trimester with systolic blood pressure [44]. In our study, maternal plasma MDA levels were not associated with risk of PE, despite its association with blood pressure. This could be attributed to MDA being a ubiquitous oxidative stress marker and hence fails to show any specific associations.

Conclusion

Our findings suggest that oxidative stress in early pregnancy does not play a role in the pathophysiology of PE but is more likely a consequence of preeclampsia.

Data Availability All data supporting the findings of this study are available within the paper.

Declarations

Ethics Approval The study protocol was approved by the Institutional Ethics Committee, Bharati Vidyapeeth Deemed University (IEC/2015/37, dated 03.10.2015).

Consent to Participate Written informed consent was obtained from all individual participants included in the study.

Consent to Publish The authors affirm that human research participants provided informed consent for publication of their data.

Conflict of Interest The authors report no conflict of interest.

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